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21NOV03 E853827-1 D00016
P01/7700 0.00-0327046.9

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1. Your reference — JHB/03463/JLH — 0327046.9

2. Patent application number *(The Patent Office will fill in this part)* — 20 NOV 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

QUEEN MARY & WESTFIELD COLLEGE
Mile End Road
London E1 4NS

Patents ADP number *(if you know it)* 61920330 01

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — Regulation of Sperm Function

5. Name of your agent *(if you have one)* — BROOKES BATCHELLOR LLP

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

102-108 CLERKENWELL ROAD
LONDON
EC1M 5SA

Patents ADP number *(if you know it)* ~~08142291001~~ 8835276001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
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Yes

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Continuation sheets of this form	
Description	9
Claim *(s)*	2
Abstract	-
Drawing *(s)*	-

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Priority documents	-
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	-
Request for preliminary examination and search *(Patents Form 9/77)*	-
Request for substantive examination *(Patents Form 10/77)*	-
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11. I/We request the grant of a patent on the basis of this application.

Signature [signature] Date 19 November 2003

12. Name and daytime telephone number of person to contact in the United Kingdom: John Blake 01892 510600

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REGULATION OF SPERM FUNCTION

This invention is concerned with the regulation of sperm function. In particular this invention relates to the use of specific proteins, such as fibronectin and angiotensin II, to respectively conserve sperm in a non-capacitated or non-activated state and to convert non-capacitated/activated sperm to the capacitated/activated state.

The physiological factors which induce and maintain mammalian sperm maturation and motility generally remain unclear, although several agents are known to be involved. For example, motility data on stimulated and unstimulated sperm from volunteers and patients attending fertility clinics showed that angiotensin II may increase both the percentage of motile sperm and their linear velocity, while the specific ATI receptor antagonist losartan inhibits the action of angiotensin II on the percentage of motile sperm. It has been demonstrated that angiotensin II has actions on specific motility parameters, including curvilinear velocity, straight line velocity, and amplitude of lateral head movement. These motility changes are characteristically associated with sperm capacitation, that is the capacity, eventually, to fertilize ova. These findings are the basis for the invention of International application No: PCT/GB95/0 1202 entitled "Use of angiotensin II to promote fertility".

At the same time, interest has been drawn to the interaction of sperm with tissue matrix proteins, including particularly fibronectin. It inhibits sperm motility, and its presence on sperm may be associated with infertility . In other studies, the problems associated with sperm freezing have been discussed. Sperm freezing is an essential tool in sperm preservation for artificial insemination and in vitro fertilization in both domestic animals and in humans. However, depending on species and individual, there are varying degrees of damage that are associated with sperm freezing and thawing, and fertility is impaired compared with fresh unfrozen sperm. In part this is because freezing and thawing appears to elicit changes that reflect capacitation.

This invention is based on the finding that matrix proteins such as fibronectin can be used to conserve sperm in a non-capacitated state, and that angiotensin II and peptides containing the RGD tripeptide can be used to restore capacitation.

In a first aspect the present invention comprises the use of one or more matrix proteins as an agent to conserve sperm in a non-capacitated/inactive state. The matrix protein may be added, suitably in conventional extender media, to fresh sperm for cold storage or before freezing for cryo-preservation for frozen sperm

In a second aspect the present invention comprises the use of angiotensin II or related peptides as an agent for capacitation of sperm samples that have been conserved in a non-capacitated state.

The use of the second aspect advantageously follows on from use of a matrix protein as an agent in the first aspect of this invention. The matrix protein-containing samples may result from thawing matrix protein-containing frozen sperm, or from matrix protein-containing sperm samples that have been stored unfrozen, or from samples that naturally contain a matrix protein.

Sperm that are naturally in a non-capacitated state have the inherent potential eventually to proceed to capacitation, whether stimulated or not. However freezing and thawing sperm is quite damaging to them, but those that are recovered in viable condition after thawing often proceed rapidly to capacitation, frequently limiting their usefulness post-thawing. A feature of the present invention is to make use of added matrix protein and angiotensin II to offer some control over the process, in effect providing a "brake and accelerator" under control of the technician using the sperm, for example in fertilisation studies or in treatment of patient samples in human fertility clinics or in artificial insemination of animals.

The matrix protein is typically fibronectin, but this may be replaced by other matrix proteins capable of binding to cell surfaces so as to maintain sperm in a non-capacitated state.

As a capacitating agent, angiotensin II can be replaced by related peptides such as the salts and analogs mentioned in WO 95/32725, the disclosure of which is incorporated herein by reference. A suitable analog is angiotensin II amide.

Alternatively peptides containing the tripeptide motif RGD (Arg-Gly-Asp) may be used as agents for capacitation. This motif may be provided by the tripeptide RGD itself or by other small peptides, such as the commercially available tetrapeptide RGDS (Arg-Gly-Asp-Ser). RGD is the tripeptide motif found in all the matrix proteins, to which the integrins in the cell membrane bind. Use of free RGD therefore competes with the matrix proteins, and will inhibit cell binding by the proteins.

RGD is suitably used in combination with angiotensin II, because the RGD inhibits matrix protein binding, increasing the effectiveness of added angiotensin II in stimulating capacitation/activation. However angiotensin II is an effective capacitation agent in the absence of RGD.

For storage, fresh sperm is preferably added to a sperm extender medium. Accordingly a further aspect of the invention is a sperm extender medium containing a matrix protein such as fibronectin

A sperm extender medium is frequently prepared by the user by adding a solid, typically powdered, extender composition to water. Accordingly the present invention also provides a sperm extender composition containing a matrix protein such as fibronectin.

In a still further aspect the present invention provides use of one or more matrix proteins for the preparation of an agent for conserving sperm in a non-capacitated

state or for preparation of a sperm extender medium for conserving sperm in a non-capacitated state.

When the extender medium is used for cryo-preservation, the storage medium also preferably also contains one or more cryo-protective agents such as glycerol.

Similarly, for capacitating sperm, a sperm sample may be added to a storage medium containing a capacitation agent. Accordingly the present invention also provides a sperm extender medium or composition containing angiotensin II and/or RGD.

In a still further aspect the present invention provides use of angiotensin II and/or RGD for the preparation of an agent for capacitating sperm or for preparation of a sperm extender medium or composition for capacitating sperm.

Sperm extender compositions generally contain buffers, for example citric acid or sodium citrate and sodium bicarbonate, and/ or nutrients, for example sugars such as glucose. When the sperm is to be stored frozen, then cryo-protectants such as glycerol or egg or milk proteins may be added. Antibiotics may also be added. The pH of the medium is generally about 7-8.

In general, the procedures of this invention will make use of reproductive cell media which are commercially available or well known in the art – see for example the materials listed in WO 03/072707, the disclosure of which is incorporated herein by reference.

This invention is primarily concerned with mammalian sperm, especially human sperm for use in fertility clinics and animal sperm for use in stock-breeding by artificial insemination.

A major problem with both human and domestic animal artificial insemination procedures is the timing of sperm capacitation to ovulation. Partly because of this,

success rates are often very low. By using "brake and accelerator" agents in accordance with the present invention, artificial insemination procedures can be made more precise. In a suitable extender medium, sperm can be transferred at the right state of capacitation at precisely the right time. In some circumstances, the use of a matrix protein "brake" may be a sufficient intervention, and the sperm can be capacitated by the natural release of angiotensin II that accompanies ovulation. This extends the application of the invention, by avoiding therapeutic intervention for timing of ovulation.

A further development of the invention provides both sperm enhancement and inhibition (fertility enhancement and contraception) by the use of internally applied pessaries, containing matrix protein (e.g. fibronectin) or angiotensin II (or analog) shortly before intercourse.

Accordingly the invention provides in a further aspect provides:
a sperm inhibition composition comprising one or more matrix proteins, preferably dispersed in a pessary base;
a sperm enhancement composition comprising angiotensin II or a related peptide, preferably dispersed in a pessary base.

Suitable pessary bases for human and animal use are commercially available and well known in the art. Typically they comprise fats or waxes that melt at appropriate temperature for internal use. Foam pessaries are also usable to assist in dispersion of the active substance.

The invention is illustrated, by way of example only, in the following experiments.

In the accompanying drawings:
Figure 1 is graphical representation of the results of Experiment 1
Figure 2 is graphical representation of the results of Experiment 2
Figure 3 is graphical representation of the results of Experiment 3

Materials

Bull semen sample (Semex)

Fibronectin (250µg/ml, Sigma)

RGDS (150µl/ml) (as a source of RGD)

Angiotensin II (10^{-9} moles/L)

Sp-TALP (Tyrode's Albumin Lactate Pyruvate) Media (TL, BSA, Fract V, Pyruvate, gentamicin) - a reproductive cell medium

Thermoplate - a heated microscope stage

Experiment 1

Straws of bovine sperm, were thawed in warm water bath at 37°C for 30 seconds. Samples of thawed sperm were incubated with additives as follows:

1. Sperm were incubated with 10% fibronectin (w/v) at 37°C in water bath for 10 min.

2. Sperm were incubated with fibronectin 10% plus RGDS ($5x10^{-6}$ moles/L) for 5 min. 3. Angiotensin II (10^{-9} moles/L) was added to the 10% fibronectin plus RGDS sample and incubated for further periods of 10, 20, 30 and 40 minutes.

All incubations were carried out at 37°C, using water bath and heated stage.

Semen samples (10µl) were applied to preheated (37°C) slides and placed on the microscope heated stage and examined. The number of motile sperm were counted in the control sample at zero time, and in the treated samples after the incubation times indicated.

The results are shown in Figure 1 in which the columns show motility values for

1. control 0 time;
2. fibronectin 10% 10 min;
3. 10% fibronectin + RGD 10 min;
4. 10% fibronectin + RGD + Angiotensin II 10 min;
5. 10% fibronectin + RGD + Angiotensin II 20 min;
6. 10% fibronectin + RGD + Angiotensin II 30 min;

7. 10% fibronectin + RGD + Angiotensin II 40 min;

8. control after 40 min;

from which it can be seen that:

a) Control sample showed 70% motility at the time zero

b) The effects of 10% fibronectin (250μg/ml) concentration on the sperm showed a highly significant change in total sperm motility. After incubation of 5-10 minutes the sample showed only 14% sperm motility.

c) The effects of fibronectin plus RGDS after 10 minutes showed no significant difference (23%) when compared to 10% fibronectin alone for 10 minutes.

d) By addition of Angiotensin II (10^{-9} moles/L) to the sample, there was a significant change in sperm motility. Increased incubation with Angiotensin II showed an increase in sperm motility. After 10, 20, and 30-40 minutes incubation the motility was increased from 43%, 64% and 71% respectively compared to 10% fibronectin alone. Compared to the RGD plus fibronectin sample the increase was 34%, 55%, and 62% respectively.

e) The control sample showed only 28% motility after 40 minutes incubation in the water bath.

Experiment 2

Straws of bovine sperm, were thawed in warm water bath at 37°C for 30 seconds. Samples of thawed sperm were incubated with additives as follows:

1. Sperm were incubated with 10% fibronectin (w/v) at 37°C in water bath for 10 min.

2. Angiotensin II (10^{-9} moles/L) was added to the 10% fibronectin sample and incubated for further periods of 10, 20, and 30 minutes.

All incubations were carried out at 37°C, using water bath and heated stage. Semen samples (10μl) were applied to preheated (37°C) slides and placed on the heated microscope stage and examined. The number of motile sperm were counted in the control sample at zero time, and in the treated samples after the incubation times indicated.

The results are shown in Figure 2 in which the columns show motility values for

1) control 0 min;
2) 10% fibronectin;
3) 10% fibronectin + Angiotensin II 10 min;
4) 10% fibronectin + Angiotensin II 20 min;
5) 10% fibronectin + Angiotensin II 30 min;
6) Control after 30 min;

from which it can be seen that:

a) Control sample showed 79% motility at the time zero

b) The effects of 10% fibronectin concentration on the sperm showed a highly significant change in total sperm motility. After incubation of 5-10 minutes there was only 8% sperm motility.

b) By addition of Angiotensin II (10^{-9} moles/L) to the sample, there was a significant change in sperm motility. At the time 10, 20, and 30-40 minutes from the start of incubation the motility was increased to 64%, 62% and 62% respectively compared to 10% fibronectin alone.

c) The control sample showed only 20% motility after 30 minutes incubation in the water bath.

Experiment 3

Straws of bovine sperm, were thawed in warm water bath at 37°C for 30 seconds. Samples of thawed sperm were incubated with additives as follows:

1. Sperm were incubated with RGDS ($5x10^{-6}$ moles/L) at 37°C in water bath for 5 min.
2. Fibronectin [10%] was added to the RGDS sample and incubated for a further 5 min.
3. Angiotensin II (10^{-9} moles/L) was added to the fibronectin + RGDS sample and incubated for further periods of 10, 20, and 30 minutes.

All incubations were carried out at 37°C, using water bath and heated stage.

Semen samples (10µl) were applied to preheated (37°C) slides and placed on the heated microscope stage and examined. The number of motile sperm* were counted

in the control sample at zero time, and in the treated samples after the incubation times indicated.* for assessment of motility see Vinson, G.P., Puddefoot, J.R., Ho, M.M., Barker, S., Mehta, J., Saridogan, E. and Djahanbakhch, O. (1995). Type 1 angiotensin II (AT1) receptors in sperm. Journal Of Endocrinology, **144**, 369-378.

The results are shown in Figure 3 in which the columns show motility values for

1) control 0 min;
2) pre-incubation with RGD 5 min;
3) RGD + fibronectin 5 min;
4) fibronectin + RGD + Angiotensin II 10 min;
5) fibronectin + RGD + Angiotensin II 20 min;
6) fibronectin + RGD + Angiotensin II 30 min;
7) control 30 min;

from which it can be seen that:

a) Control sample showed 62% motility at the time zero.

b) Pre-incubation of RGDS with sperm showed 19% decrease in sperm motility.

c) The effects of 10% fibronectin (250μg/ml) concentration on the pre-incubated RGDS with sperm showed a highly significant change in total sperm motility. After incubation of 5-10 minutes there was a 47% and 28% decrease of motility compared to the control and RGD treated samples respectively.

d) By addition of Angiotensin II (10^{-9} moles/L) to the sample, there was a significant change in sperm motility. The longer the incubation with Angiotensin II showed an increase in sperm motility. After 10, 20, and 30-40 minutes incubation the motility was increased from 43%, 54% and 54% respectively compared to 20% fibronectin and RGDS.

e) The control sample showed only 38% motility after 30 minutes incubation in the water bath.

CLAIMS

1. Use of one or more matrix proteins as an agent to conserve sperm in a non-capacitated state.

2. Use according to claim 1 in which the matrix protein-containing sperm is stored in liquid form.

3. Use according to claim 1 in which the matrix protein-containing sperm is stored in frozen form.

4. Use of angiotensin II or related peptides as an agent for capacitation of sperm samples that have been conserved in a non-capacitated state.

5. Use according to claim 4 in which the sperm sample that has been conserved in a non-capacitated state is obtained by thawing matrix protein-containing frozen sperm.

6. Use according to claim 4 in which the sperm sample that has been conserved in a non-capacitated state is matrix protein-containing sperm that has been stored in liquid form.

7. Use according to claim 4 in which the sperm sample that has been conserved in a non-capacitated state is a sample that naturally contains a matrix protein.

8. Use according to any one of claims 1 to 3 or 5 to 7 in which the matrix protein is fibronectin.

9. Use according to any one of claims 4 to 7 in which the capacitating agent is angiotensin II or angiotensin II amide or an oligo-peptide containing the tripeptide RGD.

10. A reproduction cell medium comprising one or more matrix proteins as an agent to conserve sperm in a non-capacitated state.

11. A reproduction cell medium according to claim 10 in which the matrix protein is fibronectin.

12. A reproduction cell medium comprising angiotensin II or a related peptide as an agent for capacitation of sperm samples that have been conserved in a non-capacitated state.

13. A reproduction cell medium according to claim 12 in which the capacitating agent is angiotensin II or angiotensin II amide or an oligo-peptide containing the tripeptide RGD.

14. A sperm inhibition composition comprising one or more matrix proteins.

15. A sperm enhancement composition comprising angiotensin II or a related peptide.

16. A composition according to claim 15 or 16 in which the active substance is dispersed in a pessary base.